ORIGINAL ARTICLE

Anti-Inflammatory Effect of Omega-3 Fish Oil Accelerates MyoD and Myogenin Expressions Following Rat Exercise-Induced Muscle Injury Model in a Dose-Dependent Manner

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ABSTRACT

Introduction: Exercise-induced muscle injury stimulates production of proinflammatory cytokines, e.g., TNF-α, resulting in impaired satellite-cell-dependent muscle regeneration. Omega-3 fatty acids emerge to possess anti-inflammatory properties. This study aims to analyze the effect of omega-3 fish oil administration on the levels of TNF-α, MyoD and myogenin expressions of satellite cells after injury. Methods: Twenty-nine adult male Wistar rats were randomized into five groups. Except for control groups, the rats underwent single bout of downhill running exercise and three groups were given low-to-high doses of omega-3 fish oil administration 2 hours after exercise. Blood samples were collected after 24 hours to measure the concentration of TNF-α using ELISA and then the soleus muscles were surgically removed after 72 hours to measure mRNA expressions of MyoD and myogenin using RT-PCR. Results: The results showed lower serum levels of TNF-α (166.83 ± 16.15 vs. 132.83 ± 25.44, 125.00 ± 17.26, 99.66 ± 32.00 pg/mL) and higher expressions of MyoD (0.47 ± 0.19 vs. 0.64 ± 0.20, 1.17 ± 0.16, 1.07 ± 0.14) and myogenin (0.45 ± 0.10 vs. 1.82 ± 0.35, 1.50 ± 0.34, 0.76 ± 0.20) in groups given low-to-high doses of omega-3 fish oil supplementation, respectively, compared to exercise group with no supplement at 72 hours after exercise. Conclusion: Our study suggests that omega-3 fish oil supplementation following muscle injury may accelerate myogenin expression and a low dose of supplementation achieves optimal effect in promoting muscle regeneration.

Keywords: Exercise-induced muscle injury, Omega-3 fatty acids, Fish oils, Satellite cells, Muscle regeneration

INTRODUCTION

Muscle injury is one of the most frequent sports injuries and causes physical incapacity. It occurs varied between 10 and 55% in sport-related injury cases (1-3). Disease, acute trauma, eccentric contraction, and exercise are all causes of skeletal muscle injury (4). Poor healing response to muscle injury can lead to prolonged healing time, recurrent injuries, and muscle fibrosis resulting in reduction of muscle mass and declined quality of life (5-7).

Inflammation plays a pivotal role in initiating the activation of skeletal muscle regeneration cascade after injury (8,9). A number of key inflammatory mediators released by infiltrated inflammatory cells are needed for new muscle formation after injury (9). Inflammation essentially involves in all stages of muscle regeneration via bridging initial muscle injury responses and muscle regeneration process (10). Previous study by Mackey et al. (11) shows that treatment with anti-inflammatory drugs which act through the inhibition of prostaglandin synthesis suppresses human skeletal muscle cell proliferation and differentiation activity. TNF-α which is produced chiefly by activated macrophages is a multifunctional proinflammatory cytokine and critical regulator of inflammatory response (9,12). TNF-α is rapidly released in blood by infiltrating macrophage after exercise-induced muscle injury and plays important roles in recovery process after muscle injury, including modulation of muscle-regulatory genes expression (13). TNF-α promotes muscle stem cells proliferation and is involved in the modulation of muscle regulatory gene MyoD (13). Eccentric exercise can lead to muscle injury and stimulates macrophage infiltration resulting in an excessive increase in TNF-α production. TNF-α has a catabolic function of muscle-specific proteins which
muscle protein degradation rate depends on its level in the blood after an injury. Low blood levels of TNF-α trigger the proliferation of satellite cells, whereas high blood levels of TNF-α stimulate myoblast apoptosis in order to avoid cell differentiation (14).

Satellite cells inhabit amongst the basal lamina and the muscle fiber sarcolemma (15). These cells are mononuclear myogenic progenitor cells responsible for muscle fiber regeneration after an injury (15,16). When a muscle damage occurs, satellite cells are initiated to drive the G0 satellite cells into G1 phase, which subsequently restart the cell cycle to proliferate, differentiate into skeletal myoblasts, and then merge with damaged myofibers for repair (16). The process of satellite cell initiation of muscle repair, proliferation, and differentiation are modulated by skeletal muscle-specific transcription factors, e.g., MyoD, myogenin, Myf5, and MRF4 (1). MyoD is re-expressed in activated satellite cells and required in the cell cycle in order to enter S phase, whereas myogenin is associated with the formation of muscle fibers through the fusion of myoblasts into myotubes (17). MyoD is expressed in the first 12 hours after muscle injury and then myogenin is detected sporadically at 48-72 hours (18).

Nutritional support is crucial to ameliorate the negative impacts of muscle injuries that result in bed rest or immobilization causing decreased levels of physical activity. Nutritional support strategies are also essential for stimulation and acceleration of tissue healing and regeneration process by modulating inflammatory responses (8,19,20). Anti-inflammatory benefits of omega-3 fish oil have garnered particular recognition in the setting of nutritional support for optimal healing and recovery phase after muscle injury (21). Omega-3 fatty acids are long-chain polyunsaturated fatty acids that predominantly include docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) contained mainly in fish oils and also plant-derived α-linolenic acid (ALA) (5). Omega-3 fatty acids are essential nutrients that cannot be produced in the human body (22). Omega-3 fish oil supplementation has been demonstrated to decrease proinflammatory cytokines after an injury (23). Furthermore, there is also some claim that omega-3 fish oil administration can improve early wound healing (8,21). In the present study, we evaluated the effects of varying doses of omega-3 fish oil (400 mg EPA dan 300 mg DHA) supplementation to satellite cell proliferation and differentiation at 72 hours after exercise-induced muscle injury.

MATERIALS AND METHODS

Animals
Twenty-nine adult male Wistar rats (2 months old), weighing 200-250 grams were utilized in this study. Rats were acquired from Unit for Animal Testing Services (UPHP), Universitas Gadjah Mada, Yogyakarta, Indonesia. This study was ratified by the Research Ethics Committee of the Integrated Research and Testing Laboratory (LPPT, Universitas Gadja Mada, Yogyakarta, Indonesia (No. 00017/04/LPPT/III/2017). Rats were caged in a standard room temperature with 12-hour light-dark cycle and given free admission to food and water. Rats were afterwards randomly ordered into 5 groups: control group with no exercise and no supplement (C1, n=5), exercise group with no supplement (C2, n=6), and exercise groups with omega-3 fish oil supplementation at 2 hours after downhill running exercise with 3 different doses: 3.6 mg/200 mg BW (C2FO1, n=6), 5.4 mg/200 mg BW (C2FO2, n=6), and 7.2 mg/200 mg BW (C2FO3, n=6).

Accclimatization procedure
Rats were acclimatized to a motorized treadmill for consecutive 7 days according to a modification of previously described method by Brown et al. (23). On the first day, the treadmill was set up with the lowest speed for 5 minutes. Rats were observed while running on a motorized treadmill, corrected when they got to the wrong directions, and gently pushed when they stopped running. After exercise, rats were then taken back to the cage and provided with drink ad libitum. On the second and third days, the treadmill was set up with the lowest speed for 20 minutes Rats were observed while running on a motorized treadmill, corrected when they got to the wrong directions, and subjected to electric foot shock when they stopped running. Rats that were exposed to electric foot shock more than 5 times in the first 10 minutes were marked and returned to the cage. On the fourth day, rats that had been marked were placed back on the treadmill with the lowest speed for 20 minutes until the seventh day. At the end of the seventh day, if rats still stopped running and exposed to electric foot shock more than 5 times in the first 10 minutes, rats were separated and assigned to control group.

VO2max prediction test
After one-week acclimatization, rats were assigned to VO2max prediction test according to a modification of previously described method by Brooks and White (24). Rats were set on a 10-percent incline treadmill with the lowest speed and then gradually increased by approximately 5 meters per minute every 3 minutes until rats became tired. The time and distance traveled were then recorded. Fatigue was determined when the rats stopped running, laid on their back or were not affected by electric foot shock 3 times at the same speed. If the rats were still trying to wake up or turn around again, indicating that the rats were not exhausted yet, they had to undergo the same procedure the next day. Rats were then grouped according to the level of VO2max.

Single bout of downhill running procedure
Exercise-induced muscle injury was performed according to previously described method by Armstrong (25). Each exercise protocol consisted of performing
downhill running on a 16-degree decline treadmill for 5 minutes followed by 2-minute rest interval until 90 minutes total duration comprising 18 repetitions.

**Omega-3 fish oil supplementation**

Rats were given single dose of omega-3 fish oil supplementation per oral with sonde at 2 hours after the single bout of downhill running with 3 different doses. C2FO1 group was administered 3.6 mg/200 mg BW, C2FO2 group was administered 5.4 mg/200 mg BW, and C2FO3 was administered 7.2 mg/200 mg BW.

**Measurement of TNF-α serum level**

Blood serum as much as 1.5 mL was collected from the retro-orbital vein at 24 hours after the exercise for measurement of TNF-as serum level. Blood was collected into a 1.5 mL Eppendorf tube, maintained at room temperature for 2 hours, and subsequently centrifuged at 2000 g and 4°C for 20 minutes. Serum samples were kept at -80°C until measurement. TNF-α serum level was measured using TNF-α ELISA kit (FineTest, Catalogue No. ERT393).

**Muscle tissue collection**

Rats were sacrificed at 72 hours after the exercise and soleus muscle tissues were harvested. Left soleus muscle tissue was extracted and processed for MyoD mRNA expression analysis, while right soleus muscle tissue was used for myogenin mRNA expression analysis. Muscle tissue samples were kept at -80°C until measurement.

**RT-PCR examination**

RNA was isolated from 20 mg of muscle tissue using GENEzol (Geneaid, Catalogue No. GZR100) as specified by the manufacturer’s instructions and subsequently quantified using a spectrophotometer. RNA was then reverse transcribed into cDNA. The mixture for cDNA amplification contained 5X RT Buffer (Toyobo, Catalogue No. TRT-101), Random Primers (Takara, Catalogue No. 3801), dNTPs (Takara, Catalogue No. 4030), and ReverTra Ace (Toyobo, Catalogue No. TRT-101). PCR was performed under the following conditions: denaturation at 30°C for 10 minutes, annealing at 42°C for 60 minutes, and extension at 90°C for 5 minutes. Gene amplification using a PCR-based method was done using the following specific primers: MyoD (forward, 5′-ACT ACA GCG GCC ACT CAG AC-3′; reverse, 5′-ACT GTA GTC GCC GCC GTG GC-3′), myogenin (forward, 5′-TGA ATG CAA CTC CCA CAG C-3′; reverse, 5′-CAG ACA TAT CCT CCA CAT TG-3′). The cDNA amplification was conducted in a 25 μL reaction volume with 12.5 mL GoTaq® Green Master Mix (Promega, Catalogue No. 7122). Amplification products were then visualized using agarose gel electrophoresis.

**Statistical analysis**

The data were presented as mean ± standard deviation. Statistical analysis was performed with using SPSS Version 22.0 software. A one-way ANOVA with post hoc analysis (Bonferroni) was conducted for comparative analysis and linear regression was conducted to determine correlation. The normal distribution of the data was examined by the Kolmogorov-Smirnov test. A p-value < 0.05 was interpreted to be statistically significant.

**RESULTS**

**TNF-α serum level**

Kolmogorov-Smirnov test showed that the data were normally distributed (p > 0.05), thus one-way ANOVA test was conducted. TNF-α serum levels were significantly increased in all exercise groups 24 hours after exercise compared to control group. TNF-α serum level was significantly lower in group given high dose of omega-3 fish oil supplementation (99.66 ± 32.00, p < 0.05), whereas insignificantly lower in groups given low-to-medium doses of omega-3 fish oil administration (132.83 ± 25.44, 125.00 ± 17.26, respectively, p > 0.05) compared to exercise group with no supplement (166.83 ± 16.15). TNF-α serum levels were reduced in a dose-dependent manner, nevertheless, there was no statistically significant difference between the groups given omega-3 fish oil supplementation (Fig. 1).

**Figure 1: TNF-α serum levels 24 hours after exercise.**

* = p < 0.05 vs. C1, # = p < 0.05 vs. C2

**MyoD mRNA expression**

Kolmogorov-Smirnov test showed that the data were normally distributed (p > 0.05), thus one-way ANOVA test was conducted. MyoD mRNA expressions were significantly higher in all exercise groups 72 hours after exercise contrasted to control group (p < 0.05). MyoD mRNA expressions were significantly higher in groups given medium-to-high doses of omega-3 fish oil supplementation (1.17 ± 0.16, 1.07 ± 0.14, respectively, p < 0.05) compared to exercise group with no supplement (0.47 ± 0.19) and exercise group given low dose of omega-3 fish oil supplementation (0.64 ± 0.20). MyoD expression level was highest in exercise group given medium dose of omega-3 fish oil supplementation. In addition, there was no statistically significant difference between exercise group with no supplement and exercise group given low dose of omega-3 fish oil supplementation (Fig. 2).
Myogenin mRNA expression
Kolmogorov-Smirnov test showed that the data were normally distributed (p > 0.05), thus one-way ANOVA test was conducted. Myogenin mRNA expressions were significantly higher in all exercise groups 72 hours after exercise compared to control group (p < 0.05). Myogenin mRNA expressions were significantly higher in groups given low-to-medium doses of omega-3 fish oil supplementation (1.82 ± 0.35, 1.50 ± 0.34, respectively, p < 0.05), whereas insignificantly higher in group given high dose of omega-3 fish oil supplementation (0.76 ± 0.20, p > 0.05) compared to exercise group with no supplement (0.45 ± 0.10). Myogenin expression levels were declined in a dose-dependent manner. In addition, there was statistically significant difference between exercise group given low dose of omega-3 fish oil supplementation and exercise group given high dose of omega-3 fish oil supplementation (Fig. 3).

DISCUSSION
Omega-3 fish oil supplementation decreases TNF-α serum level following exercise-induced muscle injury
Our study clearly shows that a single bout of downhill running exercise increases TNF-α serum level 24 hours after exercise. The results also indicate that omega-3 fish oil administration can reduce inflammatory process following exercise-induced muscle injury by decrease of proinflammatory cytokine TNF-α. Omega-3 fatty acids EPA and DHA are profoundly recognized for its anti-inflammatory effects (26). Our study results are in accordance with previous study by Sundrarjun et al. (27) showing that omega-3 supplementation decreases inflammation through suppression of the proinflammatory cytokines (e.g., TNF-α, IL-1, and IL-6) and inhibition of p55 TNF receptor resulting in decreased serum levels of TNF-α (28). Furthermore, omega-3 fatty acids inhibit the expression of NF-κB transcription factor that involves in decreasing the synthesis of adhesion molecules ICAM-1, VCAM-1, and E-selectin supporting leukocyte adherence to vascular endothelium during the inflammatory response (29,30). Previous study by Ramirez et al. (29) demonstrates that omega-3 fish oil contains resolvin and protectin which are EPA- and DHA-derived lipid mediators. These molecules induce lipoxygenase mRNA expression and activity that enhances the ability of omega-3 fatty acids as agonist ligands for peroxisome proliferator-activated receptors (PPARs) to modulate gene expressions associated with increased production of anti-inflammatory molecules (30). Increased circulating level of TNF-α after injury provokes failure of muscle regeneration through activation of NF-κB that leads to degeneration of muscle proteins (16). TNF-α is released by macrophages, NK cells, T cells, monocytes, neutrophils, and can be also produced by skeletal muscle fibers during muscle injury. TNF-α has several roles in inflammatory response, including activation of leukocyte chemotactic factors, expression of adhesion molecule expression, and modulation of other proinflammatory cytokines, such as IL-1, interferon γ (IFNγ) that play a pivotal role in the inflammatory processes (31).

Inflammation has been reported as a fundamental mediator of skeletal muscle regeneration after injury (32). At 2 hours after exercise, the injured muscles are in autogenic phase that the degradation of the cellular structure occurs through activation of calcium-dependent proteolytic system and lipolytic mechanisms. The injured muscles then enter the phagocytic phase characterized by high inflammatory response at 4-6 hours after exercise (27). Our study shows that omega-3 fish oil supplementation 2 hours after injury has lowered TNF-α serum level compared to exercise group with no supplement in a dose-dependent manner. Nevertheless, high dose of omega-3 fish oil supplementation could significantly reduce inflammation by inhibiting the release of TNF-α as proinflammatory cytokines.
compared to exercise group with no supplement.

**Omega-3 fish oil supplementation enhances MyoD and myogenin expressions 72 hours following exercise-induced muscle injury**

Satellite cells are skeletal muscle-specific stem cells in charge of regeneration upon injury (26). Satellite cells remain in the G0 state in the absence of stimuli and they re-enter the cell cycle when they are activated, then proliferate and differentiate into myoblasts to become mature myocytes and merge to injured muscle fibers (19,26). Myogenesis is controlled by expression of myogenic regulatory factors (MRFs) which determine when satellites cells are in dormant, activated, proliferative, or differentiation state (26). MyoD and myogenin are myogenic regulatory factors that play an influential role in regulating myogenesis during skeletal muscle regeneration. Deficiency of these protein lead to impaired muscle regeneration. MyoD and myogenin have been revealed to be included in the proliferation and differentiation of satellite cells (33).

In the present study, we evaluate MyoD and myogenin expressions at a single time point (72 hours) after exercise. At 72 hours after exercise, satellite cells enter the differentiation state, but proliferative state still can be observed. Our results show that MyoD expression in exercise groups with medium-to-high doses of supplement are significantly higher compared to control group and exercise group with no supplement indicating omega-3 fish oil supplementation after injury enhances MyoD expression. These results are in accordance with previous study by Peng et al. (34) that shows a significant increase in C2C12 cell proliferation given omega-3 fish oil administration. Omega-3 fatty acids increase cell proliferation by stimulating the cell cycle. A low level of DHA and EPA modulates cyclin D and E expressions that play a key role in the cell cycle. Upregulation of cyclin D expression in the beginning of the G1 phase and increased level of cyclin E at the end of G1 phase stimulate the cells to enter S phase and then proceed to the next phase (35). Other previous studies show that omega-3 fish oil supplementation containing EPA in animal model of inflammation decreases myostatin expression. Myostatin is a muscle development regulator that is expressed by the TGF-β group and stimulates cytokine release causing DNA damage, MyoD protein degradation, and myogenic growth factors, and also the presence of macrophage invasion that releases TGF-β suppressing MyoD level (37). In addition, satellite cells are a diverse population and most of satellite cells immediately differentiate into myocytes after entering proliferation cycle. However, a small population of satellite cells still proliferates to be maintained as stem cells in the skeletal muscle (36,37). Therefore, prolonged high level of MyoD expression 72 hours after exercise indicates delayed cell cycle withdrawal.

Our study shows a significant increase in MyoD expression 72 hours after exercise in exercise groups given medium-to-high doses of omega-3 fish oil supplementation compared to exercise group with no supplement. High dose of DHA and EPA administration in culture C2C12 myoblast cells leads to decreased expression of cyclin E and Cdk2 at the end of G1 phase resulting in prolonged G1 phase and not entering S phase. Decreased cyclin E expression leads to cell growth arrest resulting in decreased cell proliferation (35).

Satellite cells start to enter differentiation phase 72 hours after exercise. Our study shows a significant increase in myogenin expression 72 hours after exercise in exercise groups given omega-3 fish oil supplementation contrasted to control group and exercise group with no supplement indicating that omega-3 fish oil supplementation enhances myogenin expression. Our results are consistent with previous study by Abreu et al. (19) showing that omega-3 fatty acids administration stimulates the differentiation of culture myoblast L6 cells. Omega-3 fatty acids change membrane lipid composition and trigger cell differentiation by stimulating the phosphorylation of ribosomal protein S6 kinase beta-1 (p70S6K1) and activating mammalian target of rapamycin complex 1 (mTORC1), which are important proteins in the cell cycle (38). Another previous study by Chang and Dechkelbau (28) also shows that omega-3 fish oil administration increases satellite cell differentiation. Omega-3 fish oil contains resolvin, a derivative of EPA and DHA, that improves phagocytosis of necrotic cells without inducing proinflammatory cytokine release and macrophage M2 activation.

Our study shows that myogenin expression is lower in exercise group given high dose of omega-3 fish oil supplementation compared to other exercise groups with supplement. These results are likely due to prolonged satellite cell proliferation and inhibition of satellite cell differentiation, as shown by reduced cell differentiation marker 72 hours after exercise. In the normal muscle regeneration process, satellite cells will
be in the differentiation phase 72 hours after exercise-induced muscle injury. Low level of MyoD expression is required for the initiation of satellite cells entering the differentiation stage. MyoD stimulates p38 kinase activity to phosphorylate MEF2D gene that is necessary for transcriptional activation of the myogenin gene in the initial stage of cell differentiation (39).

**Correlation between TNF-α serum level and MyoD and myogenin expressions**

TNF-α is a well-known proinflammatory cytokine that promotes myogenesis (36). Previous study has shown that high level of TNF-α during myogenesis leads to activation of NF-κB and caspase-8 to inhibit cell differentiation (40). TNF-α maintains NF-κB activity to induce loss of MyoD mRNA (41). TNF-α can impair muscle regeneration by inducing loss of MyoD resulting in arrest of satellite cells in the proliferative stage and inhibition of satellite cell differentiation (42). TNF-α activates p38 signaling pathway, therefore, blocking of TNF-α causes downregulation of skeletal muscle differentiation marker expression, such MyoD and myogenin (10). In contrast, recent study finds that low level of TNF-α is still needed for myoblast proliferation modulated by activation of p38 mitogen-activated protein kinases (40). Another previous study has shown that neutralization of TNF-α in cell culture leads to block p38 MAPK activation, a signal key to the myoblast differentiation (43).

Suppression of inflammatory cell activation after muscle injury has a negative effect on muscle regeneration process. Previous study by Liu et al. (44) shows that depletion of proinflammatory cytokine TNF-α decreases MyoD and myogenin expressions resulting in impaired skeletal muscle regeneration. Another study shows that neutralization of TNF-α delays muscle regeneration process after traumatic injury (45). MyoD is a master regulatory gene of skeletal myogenesis (46). MyoD upregulates the expression of Cdk inhibitor p21 resulting in irreversible exit from the cell cycle and entering the differentiating stage (47). MyoD has been known to regulate its own expression and able to induce myogenin expression (46). Myogenin expression is essential for myogenic differentiation of satellite cell-derived myoblasts (48). Previous study has reported that myogenin expression begins on the 3rd day and remains elevated through 21 days after exercise (49). After satellite cell proliferation, cell cycle withdrawal is needed for the initiation of myogenic differentiation. A decline in MyoD expression might be necessary for entering differentiation stage (33). Study by Zammit et al. (50) has reported that MyoD disappears upon cell cycle withdrawal progresses. Myogenin expression lags behind MyoD correlating with cell cycle withdrawal and transition into differentiation stage (50). In the present study, MyoD expression is decreased in exercise group given low dose of omega-3 fish oil supplementation and myogenin expression, by contrast, is increased 72 hours after exercise-induced muscle injury, suggesting that low dose of omega-3 fish oil supplementation can optimally accelerate myogenin expression during muscle regeneration process.

**CONCLUSION**

Omega-3 fish oil supplementation decreases TNF-α serum level resulting in prolonged MyoD expression and delayed myogenin expression in a dose-dependent manner. Low dose of omega-3 fish oil supplementation after muscle injury efficiently leads to cell cycle withdrawal of satellite cells to initiate myogenin expression. At 72 hours after muscle injury, permanent cell cycle withdrawal is necessary for entering differentiation stage in which MyoD expression should be downregulated to induce myogenin expression. Our findings suggest that the use of nutritional supplements that have anti-inflammatory properties can alter skeletal muscle regeneration process after exercise-induced muscle injury.

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